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Toxicity of lithium on isolated heart mitochondria and cardiomyocyte: A justification for its cardiotoxic adverse effect

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Abstract

Mitochondria play an important role in myocardial tissue homeostasis; therefore, deterioration in mitochondrial function will eventually lead to cardiomyocyte and endothelial cell death and consequently cardiovascular dysfunction. Lithium (Li^+) is an effective drug for bipolar disorder with known cardiotoxic side effects. This study was designed to investigate the effects of Li^+ on mitochondria and cardiomyocytes isolated from the heart of Wistar rat. Results revealed that Li^+ induced a concentration- and time-dependent rise in mitochondrial ROS formation, inhibition of respiratory complexes (II), mitochondrial membrane potential (MMP) collapse, mitochondrial swelling, and cytochrome *c* release in rat heart mitochondria and also induced Caspase 3 activation through mitochondrial pathway, decline of ATP and lipid peroxidation in rat cardiomyocytes. These results indicate that the cardiotoxic effects of Li^+ were initiated from mitochondrial dysfunction and oxidative stress, which finally ends in cytochrome *c* release and cell death signaling heart cardiomyocytes.

KEYWORDS

Lithium, cardiotoxicity, mitochondrial dysfunction, apoptosis

1 | INTRODUCTION

Cardiotoxicity is an adverse side effect of drugs and a potentially life-threatening response to toxic compounds and pollutants.[1] The utilization of several drugs is associated with a risk of cardiovascular complications.[2] Lithium (Li^+) is a first-line treatment for bipolar disorder (BD), with a narrow therapeutic window that is toxic to humans and the environment.[3] Li^+ is known to produce a variety of cardiovascular effects in man and experimental animals.[4] These side effects are more severe during Li^+ intoxication, although they can happen at therapeutic levels of the metal. These effects include bradycardia, hypotension [5], reduced cardiac output, and cardiac arrhythmias.[6] Li^+ may also induce several electrocardiographic (ECG) changes, including dysfunction of sinus node, disturbances of atrioventricular conduction, nonspecific T-wave flattening, and reversible premature ventricular contractions. However, the effect of Li^+ on QT interval has not been fully explained [7]. Animal studies have demonstrated that Li^+ depresses the intracellular potassium (IK) concentration. Li^+ also replaces calcium. Therefore, interaction between Li^+ and IK, ICa, the sodium/calcium (Na/Ca) exchange currents and sodium/potassium (N/K) pump have been suggested. These disturbances seem to induce the above-mentioned ECG changes.[4,8,9] Mitochondria are intracellular compartments capable of Ca^{2+} uptake and release mainly through the mitochondrial uniporter and the mitochondrial Ca^{2+}/Na^+ exchanger, respectively. Therefore, mitochondria can considerably participate in Ca^{2+} homoeostasis and Ca^{2+} signaling.[10] Mitochondria also has an important role in myocardial tissue homeostasis; therefore, any deterioration in mitochondrial function will eventually leads to cardiomyocyte, myocarditis, cardiomyopathy, endothelial cell death, and consequently cardiovascular dysfunction.[11] Mitochondrial toxicity develops as a result of several mechanisms which interfere with the mitochondrial respiratory chain or inhibition of the important mitochondrial enzymes. The final phase of mitochondrial dysfunction induces loss of mitochondrial membrane potential and an increase in mitochondrial oxidative/nitrative stress, eventually leading to cell death.[12] Because of the abundance of mitochondria in cardiomyocytes, the role of mitochondria in calcium homeostasis and the close relationship linking oxidative metabolism with myocardial function and viability, mitochondrial dysfunction should often be considered as a prime suspect in cardiotoxicity, especially when cardiomyocyte survival is compromised. Despite the various findings indicating the occurrence of mitochondrial dysfunction in BD [13] and other separate evidences claiming the association of heart mitochondrial dysfunction with Li^+ consumption, toxicity of Li^+ on heart mitochondria has not been previously investigated. We therefore decided to evaluate the mitochondrial mechanisms involved in Li^+-induced

cardiotoxicity such as mitochondrial functionality, mitochondrial swelling, ROS formation, collapse of mitochondrial membrane potential (MMP), cytochrome *c* release as an initiator of apoptosis, ADP/ATP ratio, Caspase 3 activation and lipid peroxidation in mitochondria and cardiomyocytes isolated from the heart of rat.

2 | MATERIALS AND METHODS

2.1 | Materials

Li^+ carbonate and other chemicals were procured from Sigma–Aldrich (Taufkrichen, Germany). All chemicals were of the high commercial grade.

2.2 | Animals

Male Wistar rats weighing 150–200 g with access *ad libitum* to water were utilized in the experiments. The rats were kept in separate cages under artificial light with 12 h light/dark cycle, an environmental temperature of 21°C–23°C with a 50%–60% relative humidity. All experiments were performed according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran. After the animals were decapitated, their hearts were quickly extracted; the tissues were pooled and rapidly rinsed utilizing isotonic saline buffer. These samples were utilized for the isolation of mitochondria.

2.3 | Mitochondrial assays

2.3.1 | Preparation of mitochondria

Rats were decapitated and the heart was surgically removed, minced, and homogenized with a glass hand held homogenizer in ice-cold mitochondrial isolation medium (225 mM D-mannitol, 75 mM sucrose, and 0.2 mM EDTA, pH 7.4). Mitochondria were prepared from the homogenate utilizing differential centrifugation as described by Pourahmad et al., [14]. The resulting pellet was re-suspended in Tris buffer (50 mM Tris–HCl, 250 mM sucrose, 20 mM KCl, 2.0 mM $MgCl_2$, and 1.0 mM Na_2HPO_4, pH 7.4). The Coomassie blue protein-binding method was used to determine the protein concentration.[15] Li^+ carbonate was dissolved in Milli-Q water and the concentrations of Li^+ were chosen based on previous study.[16] Mitochondrial suspension was incubated with different concentrations of Li^+ (75–1000 μM) for 1 h before starting the experiments. Since toxicity of Li^+ occurs with serum concentrations $>1.5 \pm 2.0$ mmol/L in humans, none of the concentrations used in this *in vitro* study was at Li^+ blood toxic range.[17] Homogenization, as well as the following steps, must be carried out at 4°C to minimize the activation of damaging phospholipases and proteases.[14]

2.3.2 | Quantification of mitochondrial ROS level

After incubating isolated heart mitochondria with Li^+ (0, 75, 125, 250, and 500 μM) in respiration buffer containing 0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 μM EGTA, 0.5 mM $MgCl_2$, 0.1 mM KH_2PO_4, and 5 mM sodium succinate, the mitochondrial H_2O_2 production was assayed by F-2500 fluorescence spectrophotometer (HITACHI) using DCFH-DA (final concentration, 10 μM) for a period of 60 min. Excitation and emission wavelengths were 485 and 530 nm, respectively.[18,19]

2.3.3 | Measurement of complex II activity through the MTT assay

The activity of mitochondrial complex II (succinate dehydrogenase) was assayed via the measurement of MTT reduction [21]. Briefly, after incubating mitochondrial suspensions with Li^+ (0, 75, 125, 250, and 500 μM) at 30°C for 60 min and adding 0.4% MTT to the medium, samples were incubated at 37°C for 30 min. Then, the purple formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm with an ELISA reader (Tecan, Rainbow Thermo, Austria).

2.3.4 | Determination of mitochondrial membrane potential in isolated mitochondria

Rhodamine 123 as cationic fluorescent dye has been utilized for the determination of mitochondrial membrane potential.[20,22,23] Briefly, after incubating mitochondrial suspensions with Li^+ (0, 75, 125, 250, and 500 μM) at 30°C for 5 min,10 μM of rhodamine123 was added to the mitochondrial respiration buffer (220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 μM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 μM Rotenone). The fluorescence was measured via Schimadzou RF-5000U fluorescence spectrophotometer at the excitation and emission wavelength of 490 and 535 nm, respectively. Obtained data were shown as the percentage of mitochondrial membrane potential collapse (%$\Delta\Psi_m$) in all treated (test) mitochondrial groups.

2.3.5 | Determination of mitochondrial swelling in isolated mitochondria

The mitochondrial swelling, as a result of colloidal osmotic effects of solute flux in and out of the mitochondrial matrix, was measured by monitoring the absorbance at 540 nm (A_{540}) as described by Salimi et al.[24,25] Briefly, after incubating mitochondrial suspensions with Li^+ (0, 75, 125, 250, and 500 μM) at 30°C for 5 min in swelling buffer (70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM trisphosphate, 5 mM succinate, and 1 μM of rotenone), the absorbance was measured at 540 nm for 60 min with an ELISA reader (Tecan). A reduction in the absorbance indicates an increase in mitochondrial swelling.

2.3.6 | Determination of cytochrome c release from isolated mitochondria

Mitochondria were incubated in 1.5-mL Eppendorf tubes at 37°C for several periods. Inhibitor of MPT pore, cyclosporine A (Cs.A) at the final concentration of 5 μmol/L and anti-oxidant butylated hydroxyl toluene (BHT) also at the final concentration of 5 μmol/L were added 15 min before the addition of Li^+. After the incubation, tubes were centrifuged at 10,000×*g*. The supernatant contained the cytochrome *c* released from the mitochondria, and the pellet contained the mitochondrial

fraction. The concentration of cytochrome *c* was determined using the Quantikine rat/mouse Cytochrome c Immunoassay kit provided by R and D Systems, Inc. (Minneapolis, MN). Briefly, a monoclonal antibody specific for rat cytochrome *c* was pre-coated onto the microplate. One hundred micro liters (100 μL) of conjugate (containing monoclonal antibody specific for cytochrome *c* conjugated to horseradish peroxidase) and 50 μL of control and test group were added to each well of the microplate. One microgram (1 μg) of protein from each supernatant fraction was added to the sample wells. All of the standards, controls and test were added to two wells of the microplate. After 2 h of incubation, the substrate solution (100 μL) was added to each well and incubated for 30 min. After 100 μL of the stop solution was added to each well; the optical density of each well was determined via the earlier mentioned microplate spectrophotometer set to 450 nm.[26]

2.4 | Cardiomyocyte assays

2.4.1 | Cardiomyocyte isolation

Single cardiac myocytes were isolated from rat ventricles by collagenase digestion. Male Wistar rats (150–200 g) were anesthetized by ketamine and the heart extracted and placed in ice-cold isolation buffer (0.75 mM CaCl). Isolation buffer contained 20 mM sodium N-hydroxyethylpiperazine,-N-2-ethansulphonic acid (Hepes), 130 mM NaCl, 4.5 mM KCl, 5 mM MgCl, 1 mM NaHPO, 21 mM glucose, 5 mM Na-pyruvate, pH 7.25 with NaOH. The heart was perfused with isolation buffer plus 0.75 mM $CaCl_2$ at 37°C for 4 min before switching to Ca^{2+}-free buffer (isolation buffer plus 90 0mM EGTA) for 4 mm. The perfusate was then switched to "enzyme solution" containing 50 mL isolation buffer plus 50 mg collagenase (Worthington, type I), 5 mg protease (Sigma, type XIV), and 15 mM $CaCl_2$. The enzyme solution was continued for approximately 15 min until a soft tissue was obtained. The heart was then washed with isolation buffer containing $CaCl_2$ (150 mM). Ventricles were removed, minced into 10 pieces with small scissors and shaken for 5 min at 37°C in 20–25 mL $CaCl_2$ isolation buffer (150 mM). After filtration, cells were allowed to sediment in this buffer for 7 min. The supernatant was removed and cells resuspended in 0.5 mM $CaCl_2$, the process was repeated and cells were finally resuspended in medium containing DMEM (80%), M199 (20%), penicillin (200 U/mL), streptomycin (0.2 mg/mL), and ITSS (insulin transferrin-sodium selenite supplement, 0.5%).[27]

2.4.2 | Determination of the ATP/ADP ratio in isolated cardiomyocyte

Changes in the ATP/ADP ratio were utilized to differentiate modes of cell death and viability. Increased levels of ATP and reduced levels of ADP indicate proliferating cells. Conversely, reduced levels of ATP and increased levels of ADP represent apoptotic or necrotic cells where the reduction in ATP and increase in ADP are much more pronounced in necrosis versus apoptosis. Briefly, isolated cardiomyocytes were treated with various concentrations of Li^+ (35, 75, 125, and 250 μM) for 180 min in a medium containing DMEM (80%), M199 (20%), penicillin (200 U/mL), streptomycin (0.2 mg/mL), and ITSS (0.5%). Cells were washed with ice-cold PBS and suspended in buffer containing 25 mm HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 2 mm DDT, 5 mm EDTA, 1 mm PMSF, and 1 μM pepstatin A. The cardiomyocytes suspension was forced through a 25-gauge needle 10 times to crush the cells. The homogenate was centrifuged at 4°C for 1 h at 100,000×*g* and then ATP/ADP ratio was measured in homogenate with the ADP/ATP Ratio Assay kit (MAK135 sigma).

2.4.3 | Determination of Caspase 3 activation in isolated cardiomyocyte

Isolated cardiomyocytes were treated with vehicle; Li^+ (125 μM), Li^+ + Z-DEVD, Li^+ + Z-IETD, Li^+ + BHT, and Li^+ + Cs.A, in six distinct groups for 180 min in medium containing DMEM (80%), M199 (20%), penicillin (200 U/mL), streptomycin (0.2 mg/mL), and ITSS (0.5%). Cells were washed with ice-cold PBS without Ca^{2+} and Mg^{2+}, suspended in buffer containing 25 mm HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 2 mm DDT, 5 mm EDTA, 1 mm PMSF, and 1 μM pepstatin A, and were allowed to swell for 20 min on ice. The suspension was forced through a 25-gauge needle 10 times to crush the cells. The homogenate was centrifuged at 4°C for 1 h at 100,000×*g*. The Caspase 3 activation was measured by the Caspase 3 colorimetric assay kit. The Caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p nitroanilide (Ac-DEVD-pNA) by Caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. pNA has a λ_{max} at 405 nm. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

2.4.4 | Determination of lipid peroxidation in isolated cardiomyocyte

Lipid peroxidation is the degradation of lipids; it occurs due to oxidative damage and is a useful marker for oxidative stress. Polyunsaturated lipids are susceptible to oxidative attack, typically by reactive oxygen species, resulting in a well-defined chain reaction with the production of end products such as malondialdehyde (MDA). Cardiomyocytes were treated with various concentrations of Li^+ (75, 125, and 250 μM) and Li^+ + BHT for 180 min in a medium containing DMEM (80%), M199 (20%), penicillin (200 U/mL), streptomycin (0.2 mg/mL), and ITSS. Cardiomyocytes lipid peroxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU® -7 spectrophotometer after treating 1 mL aliquots of cardiomyocyte suspension (10^6 cells/mL) with trichloroacetic acid (70%, w/v) and boiling the suspension with thiobarbituric acid (0.8%, w/v) for 20 min.

3 | RESULTS

3.1 | Mitochondrial assays

3.1.1 | Succinate dehydrogenase activity

Mitochondrial succinate dehydrogenase (complex II) activity was assessed by the MTT test after 1 h incubation of isolated heart

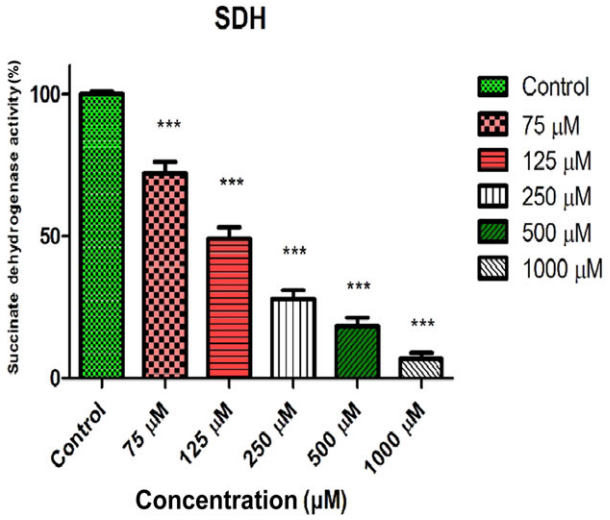

FIGURE 1 Effect of the Li^+ on succinate dehydrogenase activity in rat heart mitochondria. SDH activity was measured using MTT dye as described in *Materials and Methods*. Isolated mitochondria (0.5 mg/mL) were incubated within 1 h with various concentrations of Li^+ concentration (75,125, 250, 500, and 1000 μM). Values were expressed as mean ± SD of three separate determinations using the one-way ANOVA test, followed by the post-hoc Tukey test. $^{***}P < 0.001$ compared with untreated control mitochondria.

mitochondria with different concentrations of Li^+ (75, 125, 250, 500, and 1000 μM). As shown in Figure 1, a significant decrease in the mitochondrial metabolism of MTT to formazan ($P < 0.001$) was seen after following addition of different concentrations of Li^+. As shown in Figure 1, Li^+ (500 and 1000 μM) caused a significant decrease in the enzyme activity.

3.1.2 | ROS formation assay

As shown in Figure 2A, Li^+ (75, 125, and 250 μM) induced significant increase at ROS (H_2O_2) formation in isolated heart mitochondria. As demonstrated in Figure 2A, Li^+-induced mitochondrial ROS production was time and concentration dependent. A more substantial increase in mitochondrial ROS formation was observed in higher concentrations of Li^+ (125 and 250 μM).

3.1.3 | MMP assay

The uptake of the cationic fluorescent dye, rhodamine 123, has been used for the measurement of mitochondrial membrane potential collapse. As shown in Figure 2B, different concentrations of Li^+ (75, 125, and 250 μM) significantly decreased the MMP in a concentration and time dependent manner ($P < 0.0001$). $CaCl_2$ (100 μM), a known inducer of mitochondrial permeability transition (MPT) was used as a positive control (data not shown).

3.1.4 | Mitochondrial swelling

The decreased absorbance at 540 nm (A540) was used as indicator in mitochondrial swelling assay which is a criteria of mitochondrial membrane permeability. Addition of different concentrations of Li^+ (75, 125, and 250 μM) to isolated mitochondrial suspensions leads to mitochondrial swelling in a concentration and time-dependent manner (Fig. 3A).

3.1.5 | Cytochrome c assay

Our results showed that Li^+ is significantly caused mitochondrial swelling and collapse of the mitochondrial membrane potential. These events could result in mitochondrial permeability transition and release of cytochrome *c* from mitochondria into the cytosolic fraction. As shown in Figure 3B, Li^+ (125 μM) induced significant ($P < 0.05$) release of cytochrome *c* on the rat heart mitochondria. Significantly, the pretreatment of Li^+-treated mitochondria with the MPT inhibitor, Cs.A and BHT, an antioxidant, inhibited cytochrome *c* release compared with the sole treatment of Li^+ (125 μM) ($P < 0.05$), indicating the role of oxidative stress and MPT pore opening in cytochrome *c* release.

3.2 | Cardiomyocytes assessment

3.2.1 | Cardiomyocyte ATP/ADP ratio

As shown in Figure 4A, Li^+ in applied concentrations (35, 75, 125, and 250 μM) significantly decreased ATP/ADP ratio ($P < 0.05$) in rat cardiomyocytes.

3.2.2 | Caspase 3 assay

As shown in Figure 4B, Li^+ (125 μM) significantly increased the activity of apoptosis final mediator, caspae-3 in cardiomyocytes. To clarify the upstream mechanism involved in Li^+-induced Caspase 3 activation, we investigated the pretreating effect of Z-IETD a Caspase 8 inhibitor and Cs.A, an MPT pore sealing agent and BHT, a ROS scavenger on Li^+-treated cardiomyocytes. Our results showed that only Cs.A and BHT but not Z-IETD prevented Li^+-induced Caspase 3 activation ($P < 0.001$) suggesting that Li^+ activates a ROS-mediated mitochondrial intrinsic pathway in cardiomyocyte which could end in apoptosis.

3.2.3 | Lipid peroxidation

As shown in Figure 5, a significant amount of TBARS was formed after addition of Li^+ (75, 125, and 250 μM) into rat cardiomyocytes. The TBARS concentrations markedly increased by Li^+ ($P < 0.05$) in rat cardiomyocytes at 3 h following incubation in cardiomyocytes. Li^+-induced TBARS was prevented by ROS scavenger (BHT), MPT pore-sealing agents (Cs.A).

4 | DISCUSSION

Toxicity of Li^+ which diagnosed with serum concentrations $>1.5 \pm 2.0$ mmol/L, occasionally occurs at therapeutic levels in adults (usually in the elderly).[17] Concentrations higher than 3.5 mmol/L are potentially fatal and hemodialysis is the only recommended therapeutic solution.[28] Signs and symptoms for Li^+ overdose also includes cardiac dysrhythmias and acute renal failure.[29] Although the cardiotoxicity effects of Li^+ has recently been recognized, the cellular mechanisms of its cardiotoxicity is poorly understood.[5] The findings obtained from this study revealed that Li^+ has toxic effect

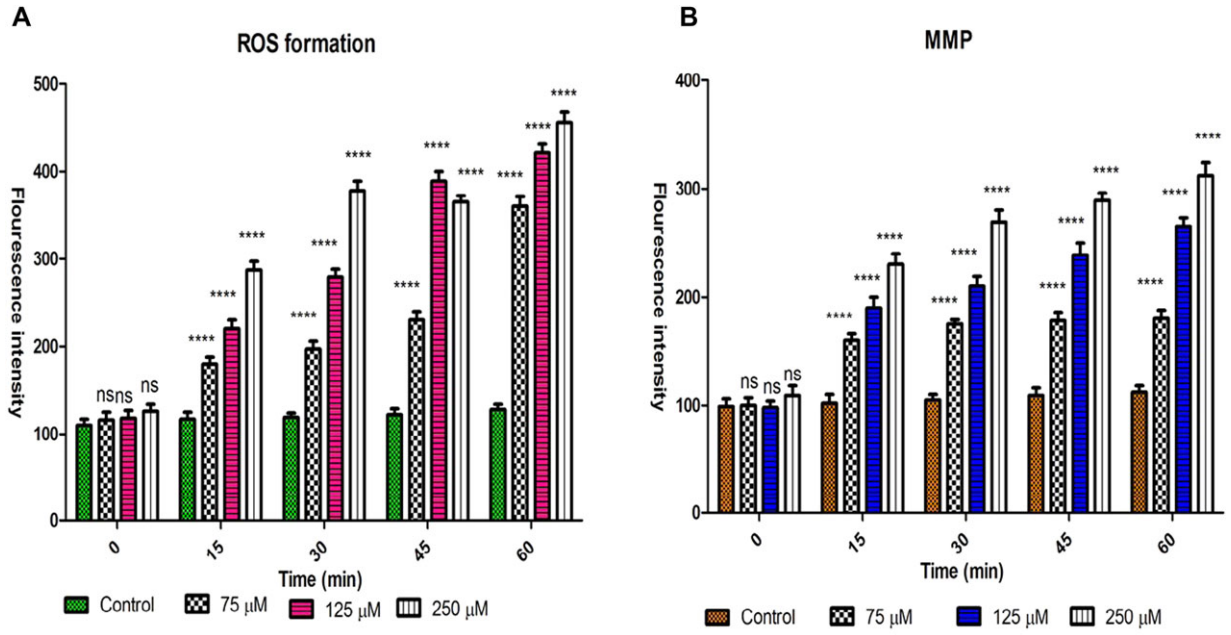

FIGURE 2 Effect of Li^+ in heart mitochondria on ROS formation (A) and MMP (B). ROS formation was evaluated after addition of Li^+ concentration (75, 125, and 250 μM) after 60 min incubation. ROS formation was determined by flourimetry using DCF-DA as described in *Materials and Methods*. The values are expressed as means ± SD ($n = 3$). $^{****}P < 0.0001$; compared with untreated control mitochondria using the two-way ANOVA test, followed by the post-hoc bonferroni test (A).
Mitochondrial membrane potential collapse ($\Delta\Psi$%) was measured by Rhodamine 123 as described in *Materials and Methods*. The effect of Li^+ concentration (75, 125, and 250 μM) on the mitochondrial membrane potential decrease in heart mitochondria was evaluated. The values are expressed as means ± SD ($n = 3$). $^{****}P < 0.0001$; compared with untreated control mitochondria using the two-way ANOVA test, followed by the post-hoc bonferroni test (B).

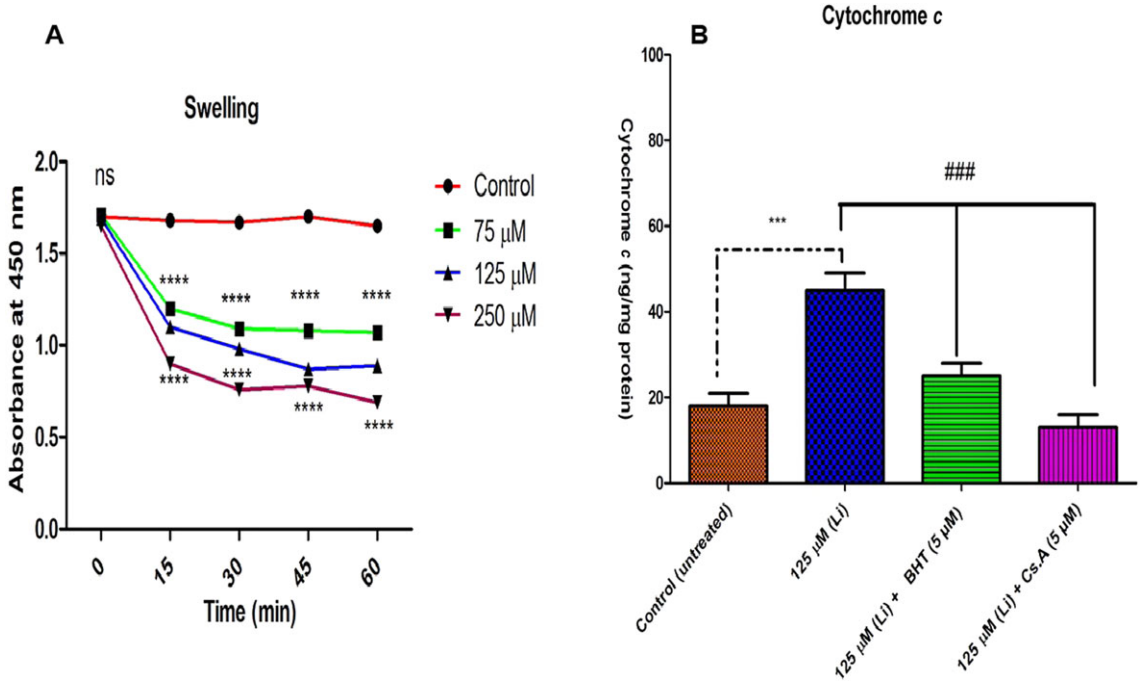

FIGURE 3 Effect of Li^+ on rat heart mitochondrial swelling (A) and cytochrome *c* release (B). Mitochondrial swelling was measured by determination of absorbance at 540 nm as described in *Materials and Methods* after addition of different concentrations of Li^+ (75, 125, 250, and 500 μM). Values represented as mean ± SD ($n = 3$). $^{****}P < 0.0001$ compared with untreated control mitochondria using the two-way ANOVA test, followed by the post-hoc bonferroni test (A).
As shown in graph B Li^+ (125 μM) induced cytochrome *c* release from isolated mitochondria and also pretreatment with BHT and Cs.A significantly inhibited cytochrome *c* release from mitochondria. The amount of expelled cytochrome *c* from mitochondrial fraction into the suspension buffer was determined using human Cytochrome c ELISA kit as described above. Values were expressed as mean ± SD of three separate determinations using the one-way ANOVA test, followed by the post-hoc Tukey test (B).

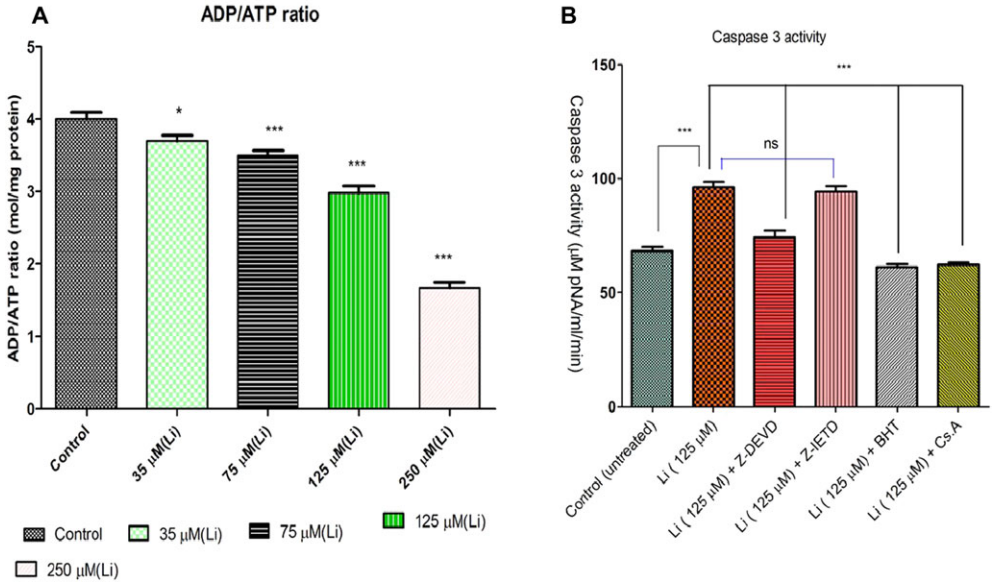

FIGURE 4 Li^+ -induced ATP decreasing in isolated cardiomyocytes (A) and Caspase 3 activity (B). ADP/ATP ratio was determined by Luciferin /Luciferase Enzyme System as described in Materials and Methods. Values are presented as mean ± SD ($n = 3$) using the one-way ANOVA test, followed by the post-hoc Tukey test. *: Significant difference in comparison with untreated control ($P < 0.05$). Rat cardiomyocyte (10^6 Cells/mL) were incubated in conventional condition (37°C and 5% CO_2-air) following the addition of Li^+. Caspase 3 activity was determined by Sigma–Aldrich kit. The kit determines produced pNA that is released from the interaction of Caspase 3 and AC-DEVD-pNA (peptide substrate). As shown in graph B, Li^+ (125 μM) significantly increased the activity of caspae-3 in cardiomyocytes. However only Cs.A (5 μM) and BHT (5 μM) but not Z-IETD (10 μM) prevented Li^+-induced Caspase 3 activation. Values are expressed as mean ± SD of three separate experiments ($n = 3$) using the one-way ANOVA test, followed by the post-hoc Tukey test. ***: Significant difference in comparison with untreated control and Li^+ treated ($P < 0.001$).

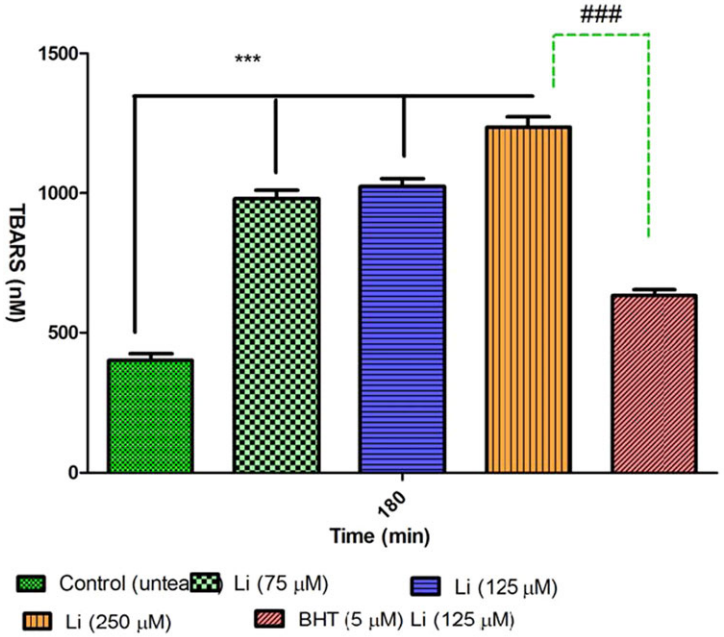

FIGURE 5 Rat cardiomyocytes (10^6 cells/mL) were incubated in at 37°C for 3 h following the addition of Li^+ (125 μM). TBARS formation was expressed as nM concentrations. Values are expressed as mean ± SD of three separate experiments ($n = 3$) using the one-way ANOVA test, followed by the post-hoc Tukey test. ***: Significant difference in comparison with untreated control cardiomyocyte ($P < 0.001$). ###: Significant difference in comparison with Li^+-treated cardiomyocyte (125 μM) and preventive agent (BHT) ($P < 0.001$).

on heart mitochondria and inhibit the activity of succinate dehydrogenase as functionality biomarker in mitochondria. Li^+ also reduced ATP in isolated cardiomyocytes (Fig. 4). Many drugs may directly or indirectly induce oxidative stress through the mitochondria via redox cycling or promoting iron accumulation and oxidative/nitrative modifications of mitochondrial proteins. This may play a crucial role in the development of myocardial dysfunction.[30] Oxidative/nitrative modification may trigger potentially harmful events, including

dissociation of catalytic subunits of enzymes, local or global unfolding, aggregation, or fragmentation, all promoting degradation of modified proteins leading to autophagy/mitophagy and endoplasmic reticulum stress.[31] The results obtained from this study demonstrate that Li^+ raises the formation of ROS in heart mitochondria. Increased ROS formation in cardiomyocytes may induce the activation of apoptotic and necrotic cell death.[32] The results obtained from this study also revealed that Li^+ activates Caspase 3 and this activation is dependent on mitochondrial pathway (Fig. 4).

Mitochondria-induced oxidative stress and the increase in mitochondrial and intracellular Ca^{2+} favor each other in a vicious relationship that amplifies damaging processes.[33] An increased availability of Ca^{2+} within mitochondria is likely to increase the accumulation of ROS by means of the processes such as increased delivery of electrons to the ETC by activating several matrix dehydrogenases [34], reduced removal of H_2O_2 resulting from a decrease in mitochondrial content of reduced glutathione (GSH) as a result of the inhibition of glutathione reductase [33], direct ETC inhibition at the level of Complex I [35]; increased formation of NO resulting in reduced activity of cytochrome oxidase [36]; binding to cardiolipin that might cause the detachment of cytochrome *c* from the IMM, thus impeding electron flow between Complex II and IV [37]; and finally PTP opening.[38] The results obtained from this study confirmed the fact that Li^+ induced increase in ROS and MPT pore opening (Figs. 2 and 3). Bioenergetic failure, enzyme inhibitions, induction of membrane disorders, the initiation of oxidative stress, as well as lipid peroxidations are being attributed to the cardiotoxic agents in mitochondria. According to our results, Li^+ also induced significant lipid peroxidation (Fig. 5).

Increased ROS generation in cardiomyocytes may trigger the activation of several mitochondrial-dependent and -independent cell death pathways involved in both apoptotic and necrotic cell death for example activation of caspases and poly(ADP-ribose) polymerases (PARP)].[39] Oxygen radical in the mitochondria may react with nitric oxide to generate a highly reactive oxidant, peroxynitrite [32,40], which may impair cellular function and lead to cell death [41] and/or dysfunction [42] Finally, the results obtained from this study indicate that Li^+ increases ROS formation in isolated rat heart mitochondria and thus leads to mitochondrial dysfunction which ends in cardiomyocytes apoptosis. Results of this study propose that nutritional consumption of antioxidant agents could reduce cardiotoxicity effects of Li^+.

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